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(54) Aglycons of A/16686 antibiotics.

(57) The invention concerns the aglycons of factors A1, A2, A3, A'1, A'2, A'3 of antibiotic A/16686, their respective tetrahydroderivatives and mixtures thereof. The aglycons are produced by selective hydrolysis of the above mentioned factors.

The compounds have antibacterial activity, in particular, against widely diffused gram positive bacteria and are particularly useful for topical treatment of wound infections and acne.

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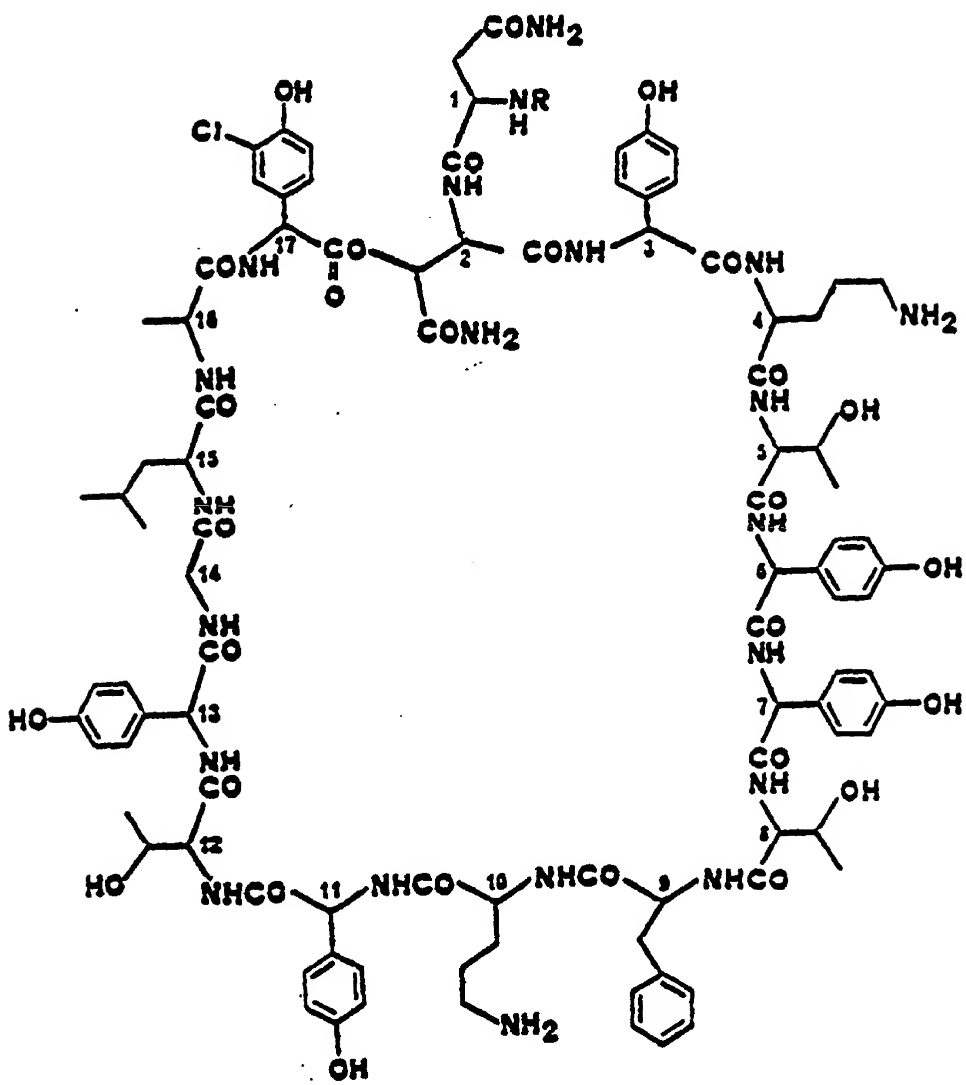
AGLYCONS OF A/16686 ANTIBIOTICS

This invention regards depsipeptidic compounds of following structure formula I



wherein:

R represents $-CO-CH=CH-CH=CH-CH_2-CH_2-CH_3$, $-CO-CH=CH-CH=CH-CH_2-CH(CH_3)_2$, $-CO-CH=CH-CH=CH-CH_2-CH_2-CH(CH_3)_2$ and the corresponding tetrahydrogenated radicals, and the acid addition salts thereof including their mixtures in any proportion, the process for their preparation and their use as antibiotics.

The above mentioned substances are correlated with antibiotic A/16686 and are produced by selective hydrolytic treatment of the compounds identified as antibiotic A/16686 factors A1, A2, A3, A′1, A′2, and A′3, their corresponding tetrahydrogenated derivatives and the mixtures thereof.

Antibiotic A/16686 is a substance active against gram-positive bacteria described in U.S. Patent 4,303,646 together with its manufacture process and the pharmaceutical compositions containing it.

It was then found that three closely related components could be isolated from antibiotic A/16686 which were named factors A1, A2 and A3. These substances as well as their preparation and uses are described

in U.S. Patent No. 4,427,656. Factor A2 is the component obtained in preponderant amount and is the most relevant for the biological activity, while factors A1 and A3 are obtained in a minor amount.

A method for selectively enhancing the production of factors A2 and/or A3 of antibiotic A/16686 by adding appropriate precursors to an A/16686 producing culture is described in European Patent Application Publication No. 259780.

European Patent Application Serial No. 88116947.8 describes antibiotic A/16686 factors A′1, A′2 and A′3 and their preparation.

European Patent Application Serial No. 88119001.1 describes the tetrahydrogenated derivatives of antibiotic A/16686 factors A1, A2, A3, A′1, A′2 and A′3 and their method of manufacture.

The compounds of this invention are also identified respectively as A/16686 factor A1 aglycon, A/16686 factor A2 aglycon, A/16686 factor A3 aglycon, A/16686 tetrahydrogenated factor A1 aglycon (formula I, R = $-CO(CH_2)_6-CH_3$), A/16686 tetrahydrogenated factor A2 aglycon (formula I, R = $-CO(CH_2)_5CH(CH_3)_2$), A/16686 tetrahydrogenated factor A3 aglycon (formula I, R = $-CO(CH_2)_6CH(CH_3)_2$).

The compounds of this invention may be produced by selective hydrolysis of a starting material selected from antibiotic A/16686 factors A1, A2, A3, A′1, A′2, A′3, a mixture of two or more of them, their respective tetrahydro derivatives and a mixture of two or more of them. Accordingly, the selective hydrolysis may be carried out either on the single factors or on any mixture of two or more of them such as, for instance, the A/16686 antibiotic complex produced by fermentation of Actinoplanes sp. ATCC 33076 (a strain which has been deposited with the permanent culture collection ATCC and is now freely available and accepted under Budapest Treaty as of January 31, 1981) as described in U.S. Patent 4,303,646. Further examples of mixtures of the A/16686 factors are those resulting from the method of European Patent Application Publication No. 259780 whereby the ratio of the factor A2 and/or A3 is selectively increased during the fermentation process, and the mixtures containing the factors of both A and A′ groups which are obtainable by fermentation of the above mentioned Actinoplanes sp. ATCC 33076 under appropriate conditions or by contacting the group A factors or a mixture thereof with the mycelium of the same strain for an appropriate period of time under proper conditions, according to the methods described in the above mentioned European Patent Application Serial No. 88116947.8.

Mixtures of tetrahydrogenated factors A1, A2, A3, A′1, A′2, A′3 are for instance obtainable by hydrogenation of antibiotic A/16686 complex as described in European Patent Application Serial No. 88119001.1.

In all cases mentioned above the starting materials may be either in the form of a free base or in the form of an acid addition salt such as those disclosed in U.S. Patents 4,303,646, 4,427,656 and in the coopending European Patent Application Serial No.88116947.8.

When the starting material consists in a mixture of two or more A/16686 factors the product mixture resulting from the selective hydrolysis may be separated into the pure components corresponding to the compounds of formula I of this invention.

The expression "selective hydrolysis" as used in this description and in the claims means an hydrolysis process carried out under controlled conditions which allows splitting the semi-acetalic bond between the sugar moiety and the rest of the A/16686 molecule without affecting the other portions of the basic structure of the A/16686 antibiotics which includes peptidic, amidic and lactonic bonds.

Accordingly, a further object of this invention is a selective hydrolysis process for the manufacture of the compounds of formula I above characterized by the fact that a starting material selected from antibiotic A/16686 factors A1, A2, A3, A′1, A′2, A′3, a mixture of two or more of them, their respective tetrahydro derivatives and a mixture of two or more of them is contacted with either:

a) trimethylsilyl iodide or trimethylsilyl chloride in the presence of sodium iodide followed by hydrolysis under mild conditions of the obtained trimethylsilyl derivative

or

b) a strong acid in the presence of a lower alkanol or a mixture of lower alkanols under anhydrous conditions.

The selective hydrolysis procedure of paragraph a) above involves the transformation of the glycosidic acetals of the A/16686 antibiotics into the corresponding trimethylsilyl ethers according to the method described by T. Morita et al. in J.C.S., Chem. Comm. 1978, page 874 and in the references cited in the same paper.

The formation of the trimethylsilyl ethers is generically carried out in the presence of an aprotic organic solvent by contacting the starting A/16686 material with a molar excess of trimethylsilyl iodide or trimethylsilyl chloride, e.g. one to three milliliters of trimethylsilyl halogenide for each gram of starting material. When trimethylsilyl chloride is used, sodium iodide is added to the reaction mixture in a proportion

ranging from 0.01 to 1 mole of sodium iodide for each mole of trimethylsilyl chloride. The aprotic organic solvent is generally selected from chlorinated lower hydrocarbons (e.g. dichloromethane, carbon tetrachloride), dimethylformamide, dimethylsulfoxide and acetonitrile and their mixtures. The temperature of the reaction is usually ranging between 10° C and 100° C, preferably between 20° C and 80° C.

As it may be appreciated by those skilled in the art, the reaction time may vary depending on the type and purity of the starting material and the specific reaction conditions of the process. In general, the reaction is completed in 0.5 to 5 hours. In any case, the reaction course may be monitored by TLC or HPLC techniques as knwon in the art. For instance, samples may be drawn at intervals and assayed in order to determine when the reaction is complete. The reaction may then be stopped in order to prevent the negative consequence of a prolonged contact of the final product(s) with the reaction mass.

The trimethylsilyl ether bonds are then easily hydrolyzed under mild conditions. The expression "mild conditions" in this case means that the reaction conditions must be appropriate for splitting the trimethylsilyl ether bond(s) without affecting the other positions of the antibiotic molecule. According to a general procedure, the reaction solution containing the trimethylsilyl derivative(s) is contacted with water or a lower alkanol or a mixture thereof at a temperature which varies from 0° C to the room temperature by keeping the pH value between 3 and 5 and for a period of time which may vary depending on the value of the pH and the temperature and usually ranges from 0.1 to 24 hours.

Accordingly, when the silylation reaction is completed, the reaction mixture is then poured into an excess of water or a lower alkanol or a mixture thereof in the presence of an approximate amount of a mild base to maintain the pH value between 3 and 5. The reaction products of formula I are then recovered from this mixture and purified according to known methods such as evaporation, extraction with solvents, precipitation by addition of non-solvents, column chromatography and the like. Sometimes, it may be convenient to concentrate the organic solution to a small volume to precipitate the crude hydrolysis product.

The isolation of the antibiotic substances of this invention from the crude hydrolysis product, their separation and purification is conducted according to known *per se* techniques which include extraction with solvents, precipitation from the obtained solution by addition of non-solvents or by changing the pH of the solution, partition chromatography, reverse-phase partition chromatography, ion-exchange chromatography, affinity chromatography, HPLC techniques and the like.

When the selective hydrolysis is carried out according to the procedure of paragraph b) above, the strong acid is usually a strong mineral acid e.g. hydrochloric or hydrobromic acid or a strong aryl or alkyl sulfonic acid such as p.toluenesulfonic acid, methanesulfonic acid, ethanesulfonic acid, their halogenated derivatives such as trifluoromethanesulfonic acid, trichloromethanesulfonic acid and the like.

The strong acid may also be a dried strong cation exchange resin in the acid form. Both gel and macroporous dried resins of this type are commercially available, e.g. Dowex[R] DR-2020, DR-2030, DR-2090 or Dowex[R] M15-DR, M18-DR, M31-DR and M32-DR. If needed, the commercially available dried resins may be further anhydrified by repeatedly washing with anhydrous methanol, by azeotropic distillation methods or by heating at 100-115° C under reduced pressure. The reaction is usually carried out in the presence of a solvent that may be selected from the organic polar solvent such as dimethylformamide or dimethylsulfoxide or, even, an excess of the same lower alkanol (i.e. a C_1-C_6 alkanol) or mixture thereof. The acid is usually dissolved in a large excess of the solvent in a concentration from 1 to 5 per cent (w/v) preferably from 2 to 3.5 per cent (w/v).

According to a typical embodiment of the method under paragraph b) above, the A/16686 starting material is added to a solution (or a suspension) of the acid in in the selected solvent in the presence of an excess of lower alkanol, preferably butanol, and the mixture is maintained at a temperature between 15 and 80° C until the splitting of the glycosidic acetal bond is completed. Also in this case, the reaction time varies depending on the type and purity of the starting materials and the specific reaction conditions. The reaction course may be monitored by TLC or HPLC techniques as indicated for the procedure according to the paragraph a) above. In general, the reaction time ranges from 0.1 to 10 hours. Also in this case, the reaction products are recovered from the reaction mixture and purified according to known methods such as those mentioned above for the procedure according to paragraph a).

When the selective hydrolysis is carried out on a substrate consisting of a mixture of two or more of the factors A1, A2, A3, A′1, A′2 and A′3 or their tetrahydro derivatives and the obtainment of the single aglycon derivatives is desired, the separation and purification of the hydrolysis product is carried out, preferably, by using column chromatography or preparative HPLC methods. The preparative HPLC operations are usually conducted under conditions which are common to the separation and purification of the A/16686 antibiotic factors. Examples of said separation and purification operations can be found, for instance, in U.S. Patent 4,427,656 where a C-18 alkyl silanized silicagel column and an eluent mixture of aqueous ammonium formate and acetonitrile is employed.

During the preparative HPLC, the eluted liquids from each injection are checked by analytical HPLC and those fractions enriched in each A/16686 factor aglycon are separated.

The fractions enriched in each of the above compounds are combined and concentrated to dryness under vacuum. The solid product(s) resulting from concentration of the eluted solution(s) is/are set free from the residual salts, then dissolved in aqueous mineral acids and the resulting solutions are freeze-dried to yield the respective pure product(s) under the form of mineral acid addition salt(s), e.g., the dihydrochloride-(s). The above operation can be repeated one or more times when the purity of the resulting products is not satisfactory. The column chromatography operations may be performed, for example, on silanized silicagel by using water:acetonitrile mixtures as the solvent and diluted hydrochloric acid : acetonitrile mixtures as the eluent. If needed, the products separated by column chromatography methods are further purified by preparative HPLC. Also in this case, it is usually preferred to isolate the final aglycon product under the form of a mineral acid addition salt by following the same procedure described above. If needed, the solid residues resulting from column chromatography or HPLC can be desalted by chromatography through a macroporous resin (e.g. XAD-2) and elution with an acidic solution.

Antibiotic A/16686 factors A1, A2 and A3 aglycons and their tetrahydro derivatives are submitted to acid/base titration, aminoacid analysis (for quantity and sequence), IR, UV, NMR spectrometry and Fast Atom Bombardment Mass Spectrometry (FAB-MS). The data resulting from these analytical tests confirm the assigned structures.

As shown in formula I the antibiotic substances of this invention possess two basic functions which can form acid addition salts according to conventional procedures.

Representative and suitable acid addition salts of the compounds of formula I include those salts formed by standard reactions with both organic and inorganic acids such as, for example, hydrochloric, hydrobromic, sulfuric, phosphoric, acetic, trifluoroacetic, trichloroacetic, succinic, citric, ascorbic, lactic, maleic, fumaric, palmitic, cholic, pamoic, mucic, glutamic, camphoric, glutaric, glycolic, phthalic, tartaric, lauric, stearic, salicylic, methanesulfonic, benzenesulfonic, sorbic, picric, benzoic, cinnamic acid and the like.

The transformation of the free amino or non-salt compounds of the invention into the corresponding addition salts, and the reverse, i.e., the transformation of an acid addition salt of a compound of the invention into the non-salt form, are within the ordinary technical skill and are encompassed by the present invention.

For instance, a compound of the invention can be transformed into the corresponding acid addition salt by dissolving the non-salt form in an aqueous solvent and then adding a slight excess of the selected acid. The resulting solution or suspension is then lyophilized to recover the desired salt.

In case the final salt is insoluble in a solvent where the non-salt form is soluble, the salt is recovered by filtration from the organic solution of the non-salt form after addition of the stoichiometric amount or a slight excess of the selected acid.

When desired, the non-salt form can be obtained from a corresponding acid salt dissolved in an aqueous solvent by neutralization to set free the non-salt form.

When, following the neutralization, desalting is necessary, a common desalting procedure may be employed.

For example, column chromatography on silanized silica gel, non-functionalized polystyrene, acrylic and controlled pore polydextrane resins (such as Sephadex LH 20) or activated carbon may be conveniently used. After eluting the undesired salts with an aqueous solution, the desired product is eluted by means of a linear gradient or a step-gradient of a mixture of water and a polar or apolar organic solvent, such as water/acetonitrile from 50:50 to about 100 per cent acetonitrile.

As it is known in the art, the salt formation either with pharmaceutically acceptable acids or non-pharmaceutically acceptable acids may be used as a convenient purification technique. After formation and isolation, the salt form of an antibiotic of formula I above can be transformed into the corresponding non-salt or into a pharmaceutically acceptable salt.

The aglycons of A/16686 antibiotics are particular]ly active against gram-positive microorganisms. The microbiological activity spectrum of antibiotic A/16686 aglycons factors A1, A2 and A3 are reported in the following Table I:

TABLE I

In vitro activity of A/16686 antibiotic aglycons (dihydrochlorides)				
Strain	MIC (mcg/ml)			
	Factor A1 aglycon	Factor A2 aglycon	Factor A3 aglycon	A/16686 factor A2
Staphylococcus aureus Tour	1	1	1	1
Staphylococcus aureus Tour [a)]	2	2	2	2
Staphylococcus aureus Tour [b)]	1	2	1	1
Staphylococcus epidermidis ATCC 12228	0.5	0.032	0.5	1
Staphylococcus haemolyticus L 602 [c)]	0.25	0.25	0.5	1
Streptococcus pyogenes C203 SKF 13400	0.008	0.032	0.008	0.063
Streptococcus pneumoniae UC41	0.016	0.032	0.016	0.063
Streptococcus faecalis ATCC 7080	1	0.5	1	1
Streptococcus mitis L 796 [c)]	0.125	0.063	0.125	0.125
Propionibacterium acnes ATCC 6922	0.063	0.125	0.25	0.25
Propionibacterium acnes L 1557 [c)]	0.063	0.125	0.5	0.25
Propionibacterium acnes L 1559 [c)]	0.125	0.125	0.5	0.25
Propionibacterium acnes L 1563 [c)]	0.125	0.125	0.25	0.25
Propionibacterium acnes L 1565 [c)]	0.125	0.125	0.5	0.25

[a)] Inoculum 10^6 cfu/ml

[b)] 30% bovine serum added

[c)] Clinical isolates

Minimal Inhibitory Concentration (MIC) is determined by either the broth (tube) or the agar serial two-fold dilution method. Culture media and growth conditions: Iso-Sensitest broth (Oxoid), for staphylcocci and Streptococcus faecalis; Todd-Hewitt broth (Difco), for other streptococcal species; Wilkins-Chalgren agar for P.acnes (T.D. Wilkins, S. Chalgren: Antimicrob. Agents Chemother. 10, 926 (1976); unless indicated otherwise, the final inoculum is of about 10^4 colony-forming units/ml or spot. MIC is read as the lowest concentration which shows no visible growth after 18-24 hours incubation at 37° C; for anaerobs the incubation is at 37° C for 48 hours in anaerobic atmosphere (N_2:CO_2:H_2, 80:10:10).

The activity of the tetrahydro derivatives of the aglycons in the same experiments are of the same level as those of the aglycons reported above. Since A/16686 aglycon factor A2 shows good activity against S.epidermidis it has been tested against a series of S.epidermidis clinical isolate pathogens of relevant clinical interest (Table II):

TABLE II

In vitro activity of A/16686 factor A2 aglycon (dihydrochloride) against selected Staphylococcus epidermidis strains (clinical isolates)		
Organism	MIC (mcg/ml)	
	Factor A2 aglycon	Antibiotic A/16686 factor A2
Staphylococcus epidermidis L 354	0.5	0.5
Staphylococcus epidermidis L 357	1	1
Staphylococcus epidermidis L 393[a)]	0.125	1
Staphylococcus epidermidis L 420[a)]	0.125	0.5
Staphylococcus epidermidis L 576	0.125	0.5
Staphylococcus epidermidis L 580[a)]	0.25	0.5
Staphylococcus epidermidis L 586	0.032	0.25
Staphylococcus epidermidis L 600[a)]	0.125	1
Staphylococcus epidermidis L 619[a)]	0.125	0.5
Staphylococcus epidermidis L 838[a)]	0.063	0.5
Staphylococcus epidermidis L 848[a)]	0.5	1
Staphylococcus epidermidis L 874[a)]	0.032	1
Staphylococcus epidermidis L 1065	0.032	0.5
Staphylococcus epidermidis L 1067	0.032	0.5
Staphylococcus epidermidis L 1378	0.25	1
Staphylococcus epidermidis L 1471	0.5	1
Staphylococcus epidermidis L 1480	0.5	1
Staphylococcus epidermidis L 1575[a)]	0.5	1
Staphylococcus epidermidis L 1578[a)]	0.125	0.5
Staphylococcus epidermidis L 1579	0.032	0.5

[a)] methicillin-resistant

Antibiotic A/16686 aglycons are active also in mice infected with Streptococcus pyogenes. In a representative experiment, groups of five mice (Charles River) are infected intraperitoneally with S.pyogenes C 203 SKF 13400. Inocula were adjusted so that untreated animals died of septicemia within 48 hours. Immediately after infection, animals are treated subcutaneously once with A/16686 factor A2 aglycon. On the 10th day the value for the ED_{50} in mg/kg is calculated by the method of Spearman and Karber (Finney, D.J., Statistical Methods in Biological Assay p. 524; C. Griffin and Co., London, 1952), on the basis of the percentage of surviving animals at each dose.

The ED_{50} shown by A/16686 factor A2 aglycon is of 0.11 mg/kg (in comparison with 0.14 for antibiotic A/16686 factor A2).

The antibiotic compounds of this invention are useful for preparing medicaments against infections primarily due to gram-positive widely diffused bacteria. In particular, the compounds of this invention are useful for topical treatment of skin and wound infections and acne.

For use as medicaments the compounds of this invention can be administered by different routes either in the form of free compounds or in the form of their addition salts with pharmaceutically acceptable acids, this latter form being preferred. For the medical uses the compounds of this invention are incorporated into pharmaceutical dosage forms suitable for oral, topical or parenteral administration such as tablets, capsules, lozenges, gelules, granules, powders, ointments, gels, liquid solutions, creams, solutions for injections, suspensions and the like. For instances, the formulations of said dosage forms can be carried out according to the general teaching of Remington's Pharmaceutical Sciences 17th Edition, 1985 Merck Publishing Company, Easton Pennsylvania. The topical route is usually the most suitable way to administer the compounds of this invention.

The dosage unit may contain from 0.01 to 99 percent preferably from 0.5 to 80 percent of active ingredient. The daily dosage may depend on several factors such as body weight, the infecting microorganism, the severity of the infection, the age of the patient, the period and the way of administration. In general,

the compounds of this invention are effective at a daily dosage ranging from about 2 mg to about 100 mg per kilogram of body weight, optionally divided into one or more administrations per day. In particular, for topical admnistration, ointments, creams, solutions, gels and lotions may have either a hydrophilic or hydrophobic base and preferably contain from 0.1 to 15 percent by weight of active ingredient. The topical dosage forms may contain also sorption promoters (see for instance: W.A. Ritschel and O.L. Sprockel, Drugs of Today, Vol. 24, pages 613-628, 1988) and preservatives. Obviously, the above dosages are only indicative and the most appropriate dosage can be adjusted in the specific cases and applications by relying on biological testings useful for determining the amount of active compound required to produced the desired effect.

The following examples have the purpose to illustrate the invention but should not be construed as a limitation of its scope.

EXAMPLES

Example 1 - A/16686 factor A2 aglycon.

To a mixture of 10 ml of dimethylformamide and 10 ml of acetonitrile, sodium iodide (50 mg) and A/16686 factor A2 (obtained according to U.S. Patent 4.427.656) (1 g) is added, followed by 2 ml of trimethylsilyl chloride. The suspension becomes clear. After heating at 75° C for 2 hours, 90 mg of sodium iodide is added, and the reaction mixture is heated for further 1 hour at 75° C.
After cooling water is added, the pH is brought at 4 with $NaHCO_3$ and the reaction mixture is extracted with butanol three times. The butanolic layer is separated and evaporated under vacuum while toluene is repeatedly added to the solution. The solid residue (1.25 g) is submitted to preparative HPLC by using the following apparatus for each portion of 250 mg dissolved in 5 ml of a mixture of water and acetonitrile 1:1 (v/v).
Instrument: the apparatus is set up by assembling a Waters mod. 590 pump, a Waters lambda-Max mod. 481LC detector set at 254 nm, and a Rheodyne injector equipped with a 5 ml loop.
Column: LiChrosorb RP-18, 10 micron, 250 mm x 50 mm (Merck)
Mobile phase: 0.05 M $HCOONH_4$:CH_3CN (60:40)
Flow rate: 30 ml/min

The operations are monitored by analytical HPLC (see Example 4.1). The group of fractions enriched in antibiotic A/16686 factor A2 aglycon are separated and combined.

Butanol is added to prevent foaming and the solvents are evaporated under vacuum. The residue (500 mg, 75% titre) is purified again by preparative HPLC with the same apparatus described above. Five portions of 100 mg are dissolved in 5 ml (each portion) of a mixture of water and acetonitrile 1:1 (v/v) and chromatographed eluting with a mixture of 0.05 M $HCOONH_4$:CH_3CN 62:38 (v/v). The fractions containing the pure component are pooled; the solvents are evaporated under vacuum while repeatedly adding butanol to obtain a residual butanolic solution (100 ml). The solution is washed with a saturated water solution of NaCl (7x50 ml) until no residual $HCOONH_4$ is present. The organic phase is concentrated under vacuum to a volume of 20 ml and the precipitate is filtered off and washed with a little butanol. The filtrate and washing are evaporated to dryness. To the solid residue dissolved in 4 ml of water, 10% HCl is added to pH 3.5 and the precipitate formed is filtered off on a Millipore [R] filter. The solution is lyophilized obtaining 120 mg of pure A/16686 factor A2 aglycon dihydrochloride.

Example 2 - A/16686 factors A1, A2, A3 aglycons.

A solution of 10 g of A/16686 complex (obtained according to U.S. Patent 4.303.646) in a mixture of 180 ml of anhydrous dimethylformamide and 60 ml of butanol, containing 2.3% (w/v) of HCl is heated at 70-75° C (bath temperature) for 2 hours with stirring. After cooling at 0° C the reaction mixture is brought to pH 4 with solid $NaHCO_3$, filtered and the butanol is evaporated under vacuum in a rotary apparatus. By adding ethyl ether a precipitate forms. After standing at -15° C overnight the surnatant is discharged and the residual solvents are evaporated under vacuum. The oily residue (6 g) is dissolved with 100 ml of a mixture of water and acetonitrile 1:1 (v/v) and charged on a chromatographic column (600 mm x 65 mm) containing 800 g of silanized silica gel (70-230 mesh) (Merck) prepared by slurring with methanol, washing with 2 liters of water, conditioning with 2 liters of 2% aqueous $HCOONH_4$, and finally washing with water.

Water (1 liter) is passed through the column, followed by 1% (w/v) HCl (2 liters). Finally, the column is eluted successively with a mixture of 1% HCl:CH_3CN, 90:10 (2 liters); 1% HCl: CH_3CN, 85:15 (2 liters) and 1% HCl:CH_3CN, 75:25 (2 liters). Fractions of 30 ml each are collected and checked by analytical HPLC (see Example 4.1). The groups of fractions containing respectively the aglycons of A/16686 factors A1, A2 and A3 are pooled; butanol is added and the solvents are evaporated under vacuum to dryness.

The portion containing aglycon factor A2 is dissolved in a few milliliters of diluted HCl and lyophilized, obtaining 1.05 g of pure A/16686 factor A2 aglycon dihydrochloride.

The portion containing aglycon factor A1 (0.9 g) is dissolved in 30 ml of water:acetonitrile, 1:1, and the solution is charged on a chromatographic column containing 800 g of silanized silica gel 60 (Merck) prepared as described above. The column is washed with 1 liter of water, then with 2 liters of 1% HCl and finally eluted with 1% HCl:CH_3CN mixtures of the following proportions: 90:10 (2 liters); 85:15 (1 liter); 80:20 (1 liter); 75:25 (1 liter).

Fractions of 30 ml are collected and checked by analytical HPLC (see Example 4.1). Fractions 250-280 containing aglycon factor A1 are pooled and evaporated to dryness after adding butanol. The solid residue (300 mg) is purified by semi-preparative HPLC using the following apparatus for each portion of 20 mg dissolved in 2 ml of a mixture of water and acetonitrile 1:1.

Instrument: Hewlett-Packard liquid chromatograph, Mod. 1080 equipped with a UV detector set at 254 nm, and a Rheodyne injector with 200 microliters loop.

Column: Hibar LiChrosorb RP 8, 7 micron, 250 mm x 10 mm (Merck)

Mobile phase: 0.05 M $HCOONH_4$: CH_3CN (55:45), pH 4

Flow rate: 5.5 ml/min

Fractions containing pure aglycon factor A1 are collected, combined and, after addition of butanol, evaporated under vacuum to dryness. The residue is dissolved in a little water and lyophilized until $HCOONH_4$ is present. The residue is re-dissolved in a few milliliters of diluted HCl and lyophilized obtaining 140 mg of pure A/16686 factor A1 aglycon dihydrochloride.

The fractions containing aglycon A3 (0.08 g) is dissolved in 30 ml of water and the solution is charged on a chromatographic column containing 800 g of silanized silica gel 60 (Merck) prepared as described above. The column is washed with 1 liter of water and eluted with a 1% HCl:CH_3CN, 75:25 mixture (10 liters).

Fractions of 30 ml are collected and checked by HPLC. Fractions 191-230 are combined and butanol is added thereto. The solvents are evaporated under vacuum and the residue is desalted, re-dissolved in diluted HCl and then lyophilized as described for factor A1, obtaining 300 mg of pure A/16686 factor A3 aglycon dihydrochloride.

Example 3 - A/16686 tetrahydro factors A1, A2, A3 aglycons.

A sample of 10 grams of mixture of A/16686 tetrahydrogenated factors A1, A2, A3, A′1, A′2 and A′3 obtained as in European Patent Appln. Ser. No. 88119001.1 is hydrolyzed for 3 hours under the conditions of Example 2. The reaction mixture is processed as in Example 2 and the tetrahydrogenated aglycons are recovered as dihydrochlorides.

Example 4 - Analytical assays and physico-chemical characterization.

4.1 - Analytical HPLC

Apparatus: Hewlett-Packard liquid chromatograph, mod. 1084 B equipped with a UV detector set at 254 nm.

Column: Erbasil C-18, 10 micron, 250 mm x 4.6 mm (Carlo Erba).

Mobile phase:

A) 0.05 M $HCOONH_4$

B) CH_3CN

Flow rate: 1.5 ml/min Gradient Profile:

min	0	15	16	28	30
% B	38	38	55	55	38

Under these conditions the retention times (t_R) are as follows:

	t_R (minutes)
Antibiotic A/16686 factor A1 aglycon	14.40 (6.82)
Antibiotic A/16686 factor A2 aglycon	19.33 (9.69)
Antibiotic A/16686 factor A3 aglycon	20.96 (12.85)
The values for the respective di-mannosylated compounds are reported in brackets.	
Antibiotic A/16686 tetrahydro factor A1 aglycon	17.40 (8.97; 10.66)
Antibiotic A/16686 tetrahydro factor A2 aglycon	20.38 (12.24; 15.33)
Antibiotic A/16686 tetrahydro factor A3 aglycon	22.65 (19.03; 20.55)
The values for the respective di- and mono-mannosylated compounds are reported in brackets.	

4.2 - Amino acid analysis and ^{1}H NMR spectra

The acid hydrolysis is performed on the A/16686 aglycons with 6N HCl at 105 °C for 20 hours. The mixture of amino acids is separated by column chromatography on a strongly acidic sulfonic divinylbenzene resin (Dowex 50 W) by eluting with aqueous HCl of increasing concentrations from 0.05N to 2N.

The amino acids are identified by comparison with authentic samples on the basis of ^{1}H NMR and GC-MS. The amino acid ratio and their sequence in the intact molecules are determined by NMR experiments.

All compounds show the same amino acids compositions and sequence.

The following Table III shows the type and number of amino acid residues in each of the aglycons.

Table III

Amino acid	Number of units
Threo-beta-hydroxyaspartic acid	1
Aspartic acid	1
Allothreonine	3
Glycine	1
Alanine	1
4-Hydroxyphenylglycine	5
Leucine	1
Phenylalanine	1
3-Chloro-4-hydroxyphenylglycine	1
Ornithine	2

Two equivalents of ammonia per mole of each aglycon is titrated in the respective acid hydrolysis mixtures by means of an amino acid automatic analyzer providing evidence of two primary amide groups. Furthermore, the total number of nitrogen atoms (19) resulting from ^{15}N NMR experiments exceeds by two the number of nitrogen atoms involved in the peptide bonds according to the number of aminoacids in the molecule (Table III) and titration of the aglycons does not show any presence of free carboxylic groups. These considerations support that the two primary amide groups are on the aspartic and threo-beta-hydroxyaspartic acid units, respectively.

The ^{1}H NMR spectra are recorded on a Brucker AM 500 spectrometer equipped with an Aspect 3000 computer at 500 MHz. The following Table IV shows the chemical shifts (delta, ppm) of antibiotic A/16686

factor A2 aglycon in D_2O:DMSO, 4:1 at pH 4.6, temperature 40 °C, internal standard TMS (delta = 0.00 ppm).

Figure 1 reports the ^1H NMR spectrum of factor A2 aglycon. The ^1H NMR spectra of the other aglycons are substantially identical to the one of the aglycon of factor A2 apart from the signals attributed to the fatty acid chains attached to the asparagine moiety. In all spectra the signals corresponding to the sugar moieties are absent. The spectra of the tetrahydro aglycons do not show the resonance signals of the vinylic protons of the fatty acids side-chains.

TABLE IV

	Amino acid	HC_{alpha}	HC_{beta}	others
1	Aspartic acid	4.70	2.25, 2.05	-
2	Beta-hydroxyaspartic acid	5.53	5.83	-
3	4-Hydroxyphenylglycine	6.21	-	Phenyl 7.44 (b,f), 6.98 (c,e)
4	Ornithine	4.22	1.97, 1.71	-
5	Threonine	4.38	4.01	HC_{gamma} 1.05
6	4-Hydroxyphenylglycine	6.78	-	Phenyl 6.75 (b,f), 6.39 (c,e)
7	4-Hydroxyphenylglycine	5.48	-	Phenyl 6.73 (b,f), 6.47 (c,e)
8	Threonine	3.74	3.92	HC_{gamma} 0.81
9	Phenylalanine	4.93	1.97, 1.71	Phenyl 7.23 (b,f), 6.94 (c,d,e)

TABLE IV (continued)

	Amino acid	HC_{alpha}	HC_{beta}	others
10	Ornithine	4.18	2.25, 1.88	HC_{gamma} 1.67, HC_{delta} 2.98
11	4-Hydroxyphenylglycine	6.88	-	Phenyl 7.26 (b,f), 6.64 (c,e)
12	Threonine	4.68	3.93	HC_{gamma} 0.94
13	4-Hydroxyphenylglycine	6.07	-	Phenyl 7.06 (b,f), 6.67 (c,e)
14	Glycine	3.79, 3.02	-	-
15	Leucine	4.36	1.47	HC_{gamma} 1.47, HC_{delta} 0.73
16	Alanine	4.22	1.45	-
17	3-Chloro-4-hydroxyphenylglycine	5.01	-	Phenyl 6.82(f), 6.53(b), 6.33(e)

fatty acid side chain: 5.58 (HC_{alfa}); 6.07 (HC_{delta}); 6.53 (HC_{beta}); 7.25 (HC_{gamma}); 1.98 (CH_2); 1.68 (HC_{zeta}); 0.85 ($2CH_3$)

4.3 - Lactone ring

The presence of a lactone ring is supported by the absorbance at 1760 cm^{-1} in the IR spectrum (see under Example 4.4). The position of the lactone bond is established by

a) identification of the amino acid contributing to the lactone bond with its carboxylic group

b) identification of the hydroxy-amino acid contributing to the lactone bond with its hydroxyl group.

According to step a) factor A2 aglycon is reduced with $Ca(BH_4)_2$ and then hydrolyzed with concentrated HCl as described in the copending European Patent Application Ser. No. 88119001.1. The presence of the 2-amino-2-(3-chloro-4-hydroxyphenyl)ethanol in the hydrolyzate confirms the position of the carboxylic moiety forming the lactone bond in the aglycons structure formula I.

According to step b) factor A2 aglycon is reacted with phenylisocyanate and then hydrolyzed as described in European Patent Application Ser. No. 88119001.1 . The constant amount of hydroxyaspartic acid versus the decrease of the other hydroxylated aminoacids confirms the position of the hydroxy group involved in the lactone bond in the aglycons structure formula I.

4.4 - I.R. Spectra

The I.R. spectrum of factor A2 aglycon recorded as nujol mull with a Perkin-Elmer mod. 580 spectrophotometer is shown in Figure 2 of the accompanying drawings. The following absorption maxima are observed: 3700-3100 (ny NH and ny OH), 3020-2800 (nujol), 1760 (ny C=O lactone), 1640 (ny C=O, amide I), 1510 (delta NH, amide II), 1460 and 1375 (nujol), 1230 (ny C-O, lactone), 840 and 815 cm^{-1} (gamma CH aromatics).

The spectra of the other aglycons do not show substantial differences.

4.5 - U.V. Spectra

The Ultraviolet Spectrum of factor A2 aglycon registered in 0.1N HCl with a Perkin Elmer mod. 320 spectrophotometer is given in Figure 3 of the accompanying drawings. The spectrum exhibits the following absorption maxima: 233 nm (E 1%, 1cm 206.7) and 271 nm (E 1%, 1cm 109.3).

The U.V. spectra of the other unsaturated aglycons do not show substantial differences. The tetrahydro aglycons exhibit absorption maxima at 232 and 275 nm.

4.6 - FAB-MS Spectra

The Fast Atom Bombardment Mass Spectra (FAB-MS) are recorded with a MS-50-TC instrument using glycerol as a matrix. Bombardment gas Xe; kinetic energy 6 keV; accelarating voltage 8kV. The low mass isotope of the protonated molecular ions (MH^+) have molecular weights of 2214.9 (factor A1 aglycon), 2228.9 (factor A2 aglycon) and 2242.9 (factor A3 aglycon). The tetrahydro aglycons protonated molecular ions (MH^+)indicate molecular weights of 2218.9, 2232.9 and 2246.9, respectively.

These data are within 0.3 dalton of the theoretical ones and are in agreement with the structures assigned.

4.7 - Elemental analysis

The elemental analysis gives the following approximate percentage composition:

	factor A1 aglycon	factor A2 aglycon	factor A3 aglycon
* C %	55.0	53.8	54.9
* H %	5.3	6.0	5.8
* N %	12.4	11.9	13.1
Cl %	4.7	5.1	4.8
Ashes %	0.5	0.3	0.7
** Weight loss %	8.4	11.2	7.9
	tetrahydro factor A1 aglycon	tetrahydro factor A2 aglycon	tetrahydro factor A3 aglycon
* C %	55.2	56.3	55.4
* H %	5.8	6.2	6.3
* N %	12.2	13.0	12.2
Cl %	4.8	4.6	4.6
Ashes %	0.5	0.2	0.2
** Weight loss %	7.3	6.8	6.5

* The sample has been previously dried at about 140° C under inert atmosphere.
** Thermogravimetric analysis

The values are in agreement with those calculated for the respective di-hydrochloride salts.

Example 5 - Representative examples of lotions, solutions and gel.

5.1 - Lotion

A 2 percent (w/w) hydroalcoholic lotion is formulated with the following ingredients (for 100 g of lotion)

Factor A2 aglycon	2.00 g
Lactic acid 90% (w/w) solution	0.107 g
Sodium hydroxide 10% (w/w) solution to pH 4	
Ethanol 96%	
	equal masses to 100 g
Purified water BP	

5.2 - Solution

Vials containing freeze-dried powder for reconstitution in normal saline to give a solution for wound infections or in a hydroalcoholic vehicle to provide lotions for topical use are prepared by dissolving respectively 10 mg, 25 mg, 50 mg and 100 mg of active aglycon in 1-2 ml of purified water BP, filling the vials and freeze drying to a residual moisture content of about 2%.

5.3 - Gel

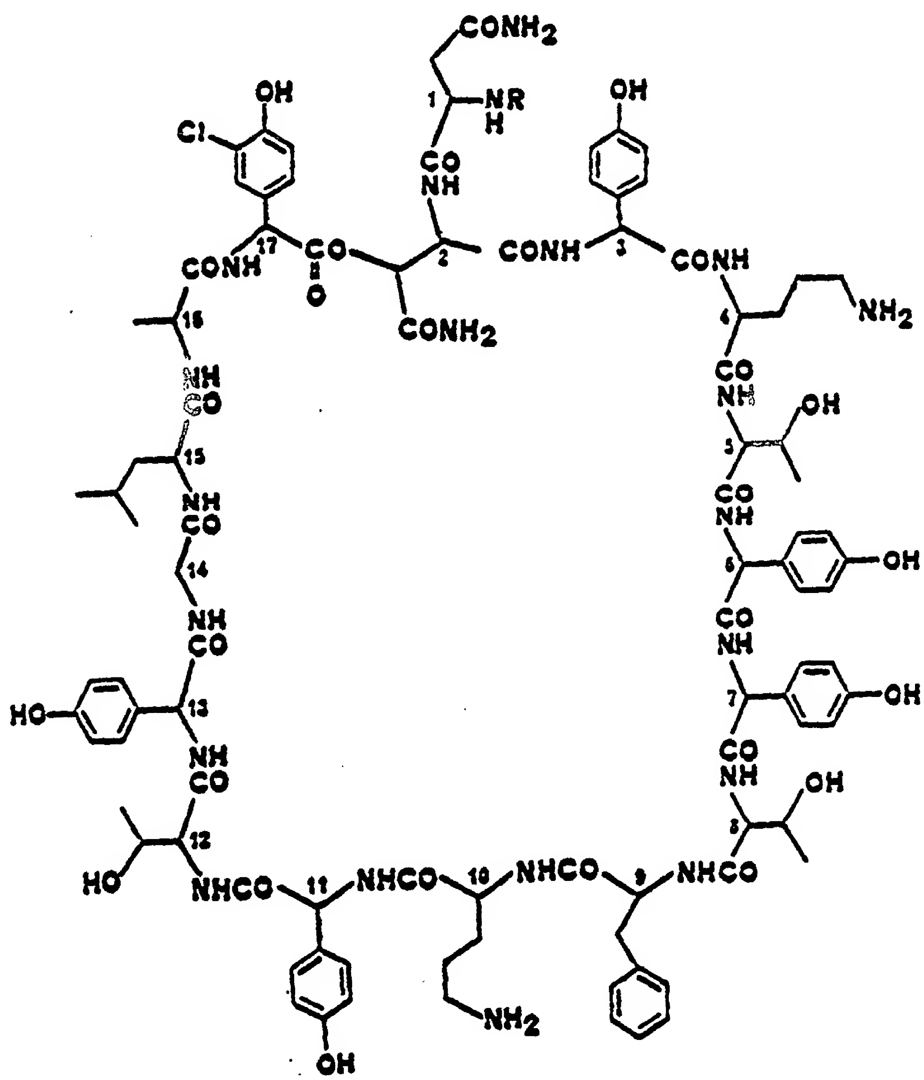
A gel topical form can be prepared with the following ingredients:

Active aglycon	2.00 g
Lactic acid 90% (w/w) solution	0.55 g
Methocel	3.00 g
Ethanol 96%	equal masses to 100 g
Purified water BP	
Sodium hydroxide 10% (w/w) solution to pH 4.0	

The active aglycon is dissolved in purified water, filtered and then the solution and the lactic acid are added at 25 °C to a methocel dispersion in the remaining water. After addition of ethanol and mixing to complete the dispersion, the pH value is adjusted to 4.0 by addition of 10% sodium hydroxide.

Claims

1) Compounds of the formula I

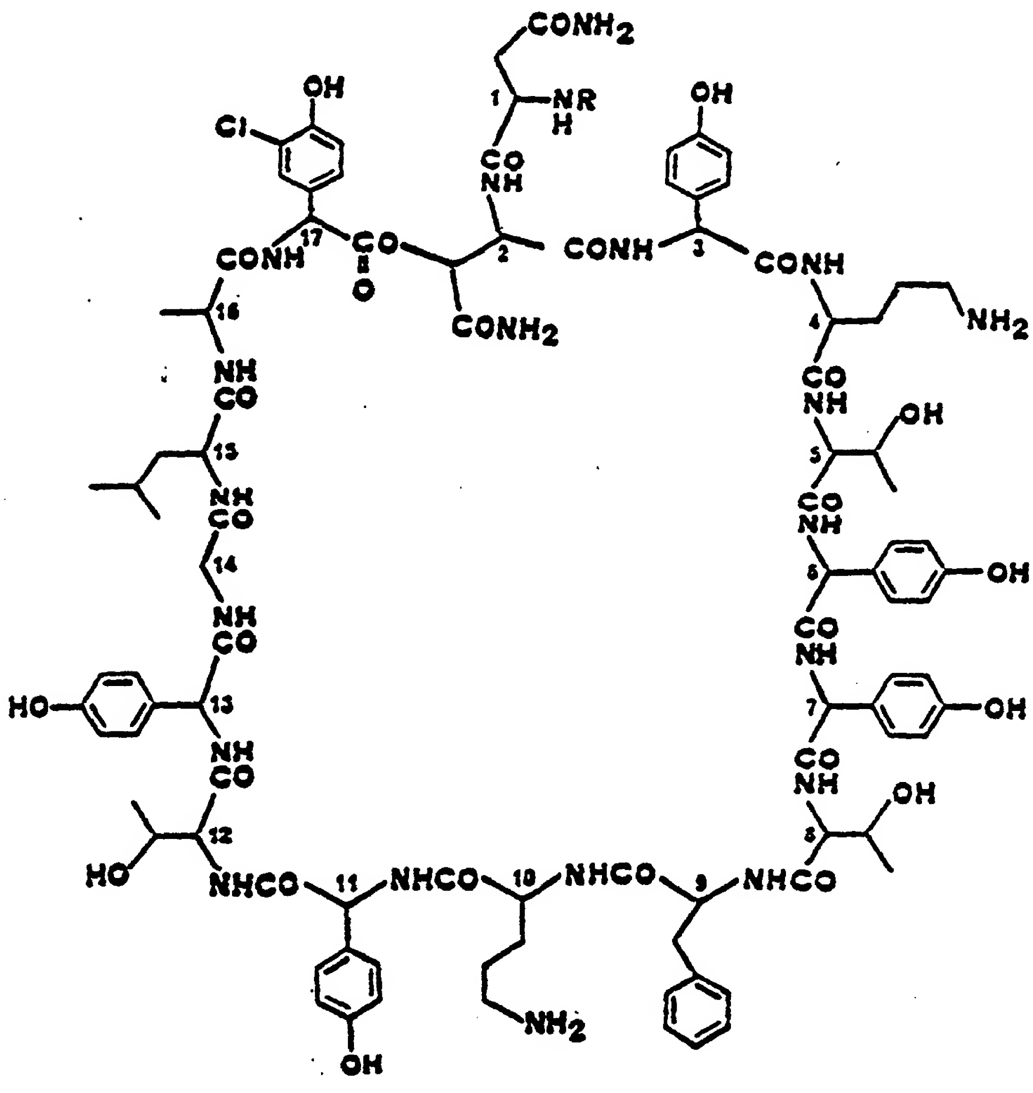


wherein
R represents $-CO-CH=CH-CH=CH-CH_2-CH_2-CH_3$,
$-CO-CH=CH-CH=CH-CH_2-CH(CH_3)_2$,
$-CO-CH=CH-CH=CH-CH_2-CH_2-CH(CH_3)_2$
and the corresponding tetrahydrogenated radicals, and the acid addition salts thereof and their mixtures in any proportion.

2) A compound of claim 1 wherein
R represents $-CO-CH=CH-CH=CH-CH_2-CH_2-CH_3$,
$-CO-CH=CH-CH=CH-CH_2-CH(CH_3)_2$,
$-CO-CH=CH-CH=CH-CH_2-CH_2-CH(CH_3)_2$ and the pharmaceutically acceptable acid addition salt thereof.

3) A compound of claim 1 wherein R represents a tetrahydrogenated radical of the formula:
$-CO(CH_2)_6-CH_3$, $-CO(CH_2)_5CH(CH_3)_2$ or $-CO(CH_2)_6CH(CH_3)_2$
and the pharmaceutically acceptable acid addition salts thereof.

4) A process for the manufacture of compound of the formula I



wherein

R represents $-CO-CH=CH-CH=CH-CH_2-CH_2-CH_3$,
$-CO-CH=CH-CH=CH-CH_2-CH(CH_3)_2$,
$-CO-CH=CH-CH=CH-CH_2-CH_2-CH(CH_3)_2$
and the corresponding tetrahydrogenated radicals, and the acid addition salts thereof and their mixture in any proportion, which comprises submitting to selective hydrolysis a starting material selected from antibiotic A/16686 factors A1, A2, A3, A′1, A′2, A′3, a mixture of two or more of them, their respective tetrahydro derivatives and a mixture of two or more of them in the form of free compounds or of an acid addition salt thereof and when a mixture of two or more compounds is used as the starting material and a pure component of the formula I is desired, separating the resulting reaction product into the single components by procedures per se known in the art.

5) A process as in claim 4 wherein the selective hydrolysis is characterized by the fact that the starting material is contacted with either

a) trimethylsilyl iodide or trimethylsilyl chloride in the presence of sodium iodide followed by hydrolysis under mild conditions of the obtained trimethylsilyl derivative

or

b) a strong acid in the presence of a lower alkanol or a mixture of lower alkanols under anhydrous conditions.

6) A process according to step a) of claim 5 wherein the reaction is carried out in the presence of an aprotic organic solvent at a temperature between 10°C and 100°C, preferably between 20°C and 80°C.

7) A process as in claim 6 wherein an excess of trimethylsilyl chloride in the presence of from 0.01 to 1 mole of sodium iodide for each mole of trimethylsilyl chloride is employed and the aprotic organic solvent is selected from chlorinated lower hydrocarbons, dimethylformammide, dimethylsulfoxide, acetonitrile and their mixtures.

8) A process as in claim 7 wherein the amount of trimethylsilyl chloride employed is one to three milliliters for each gram of starting material.

9) A process as in any of the claims 5 through 8 wherein the hydrolysis under mild condition of the obtained trimethylsilyl derivative is carried out by contacting it with water, a lower alkanol or a mixture thereof at a temperature from 0°C to the room temperature at a pH between 3 and 5.

10) A process according to step b) of claims 5 wherein the reaction is carried out in the presence of an organic polar solvent, the strong mineral acid is selected from strong mineral acids, strong aryl sulfonic acids, strong alkyl sulfonic acids, their halogenated derivatives and a dried cation exchange resin in the acid form and the lower alkanol is a C_1-C_6 alkanol, preferably, butanol.

11) A process as in claim 10 wherein the strong acid is hydrochloric acid, the solvent of the reaction is selected from dimethylformammide, dimethylsulfoxide or an excess of the same lower alkanols or mixture thereof and the reaction temperature is maintained between 15°C and 80°C.

12) A process as in any of the claims 4 through 11 and the starting material is a mixture of two or more of the factors A1, A2, A3, A′1, A′2, A′3 or their tetrahydro derivatives and the separation of the resulting hydrolysis product is carried out preferably by using column chromatography or preparative HPLC methods.

13) A compound of claim 1 for use as medicament

14) Use of a compound of claim 1 for the manufacture of an antibacterial.

15) Use of a compound of claim 1 for the manufacture of an antibacterial medicament for the topical treatment of wound infections or acne.

16) A method for combatting infectious diseases in mammals comprising administering an antibacterially effective amount of a compound of claim 1 to the mammal in need.

17) A method of claim 16 wherein the infectious disease is a wound infection or acne and the compound is topically administered.

18) A topical pharmaceutical formulation for the treatment of wound infection or acne containing an antibacterially effective amount of a compound of claim 1.

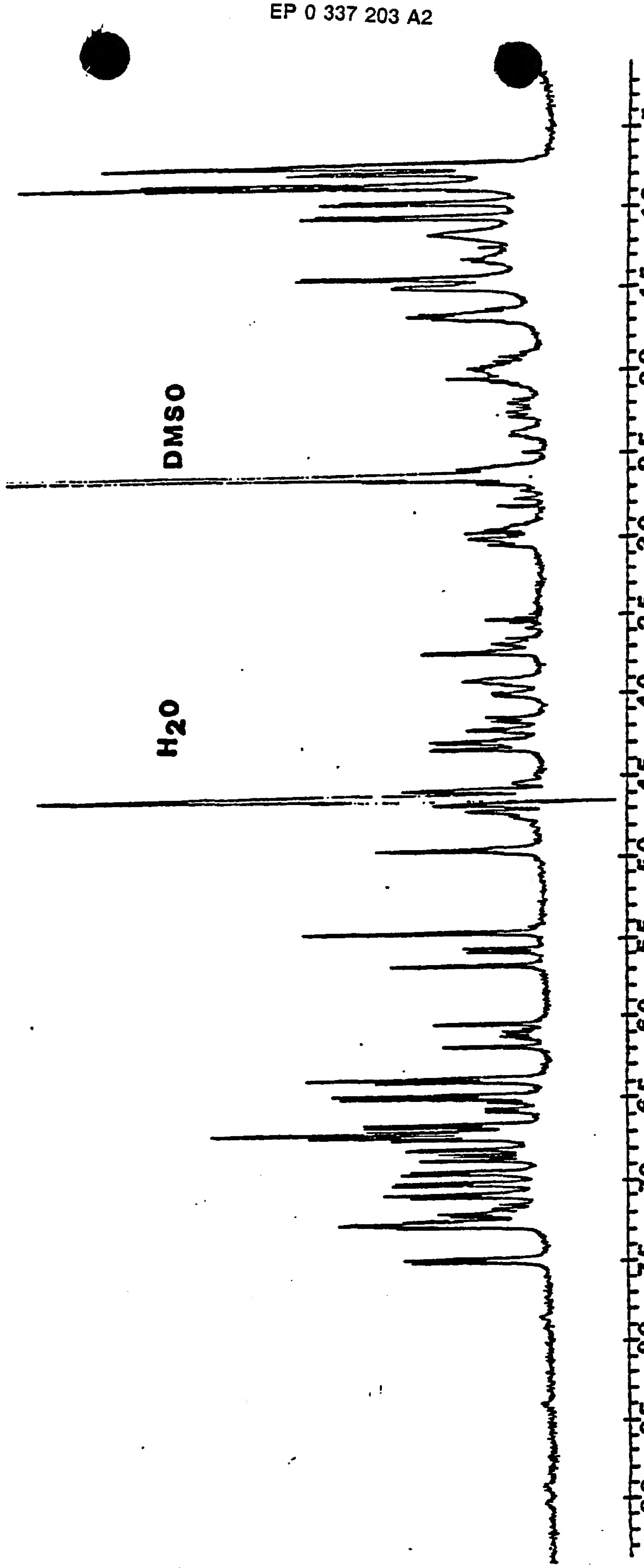


FIG. 1: ^{1}H NMR Spectrum of factor A2 aglycon

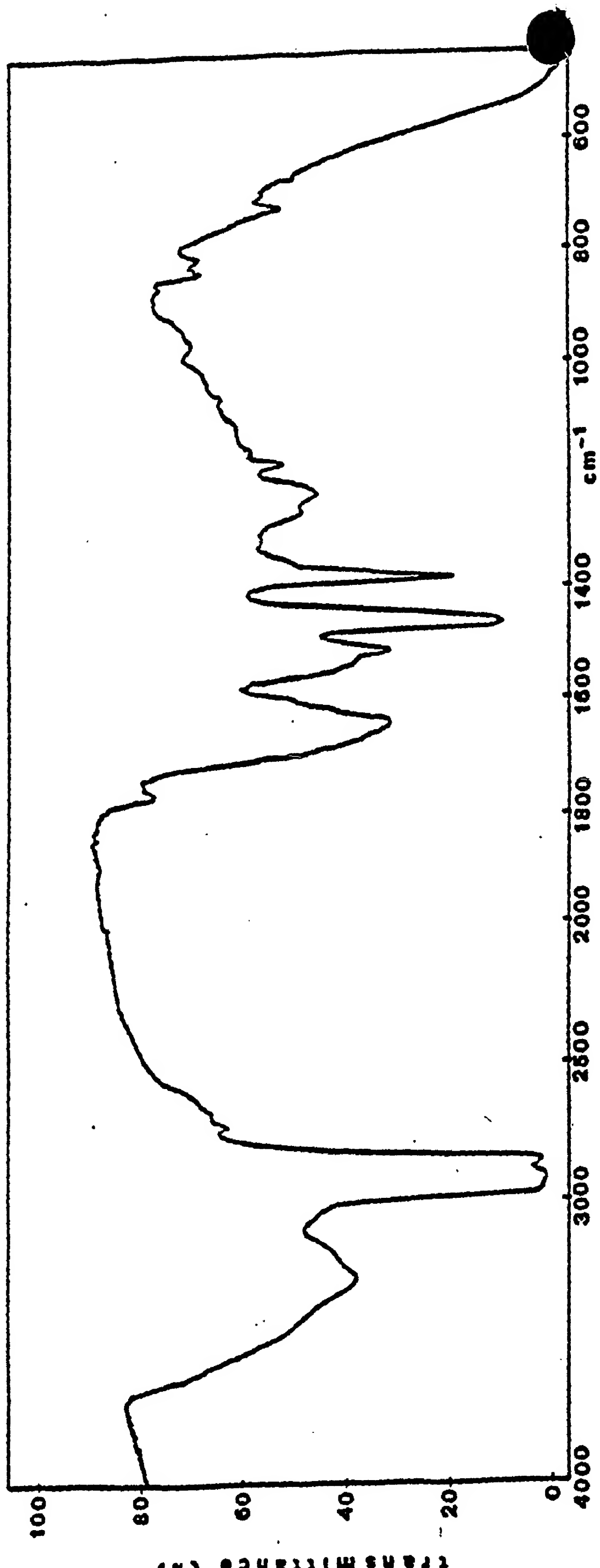


FIG. 2: I.R. Spectrum of factor A2 aglycon

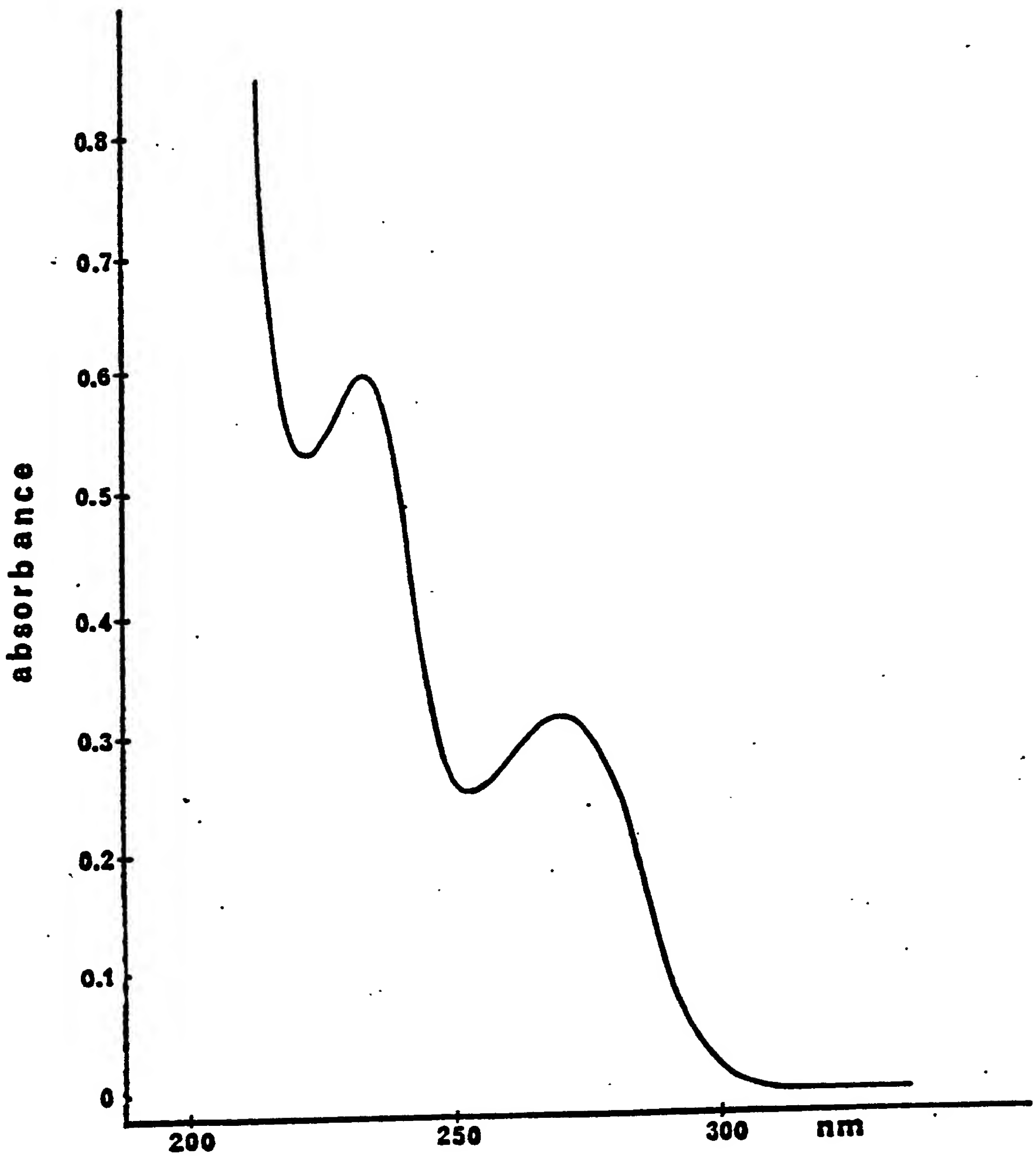


FIG. 3: U.V. Spectrum of factor A2 aglycon

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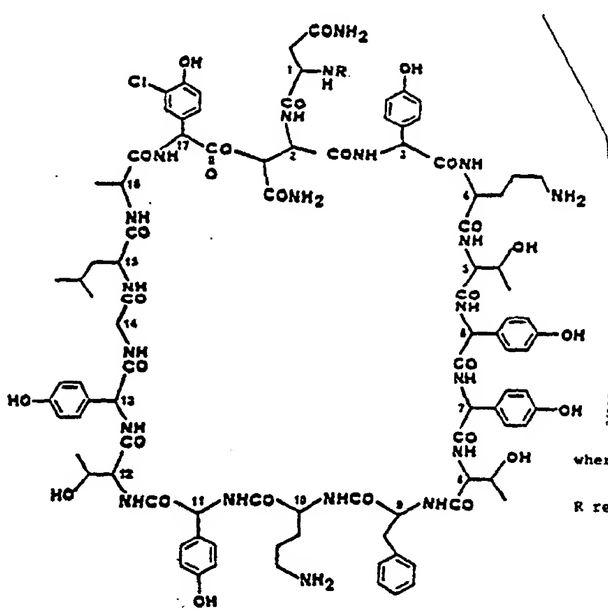
(54) **Aglycons of A/16686 antibiotics.**

(57) The invention concerns the aglycons of factors A1, A2, A3, A'1, A'2, A'3 of antibiotic A/16686 of following structure formula I



wherein:

R represents $-CO-CH=CH-CH=CH-CH_2-CH_2-CH_3$,
$-CO-CH=CH-CH=CH-CH_2-CH(CH_3)_2$,
$-CO-CH=CH-CH=CH-CH_2-CH_2-CH(CH_3)_2$,

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their respective tetrahydroderivatives and mixtures thereof. The aglycons are produced by selective hydrolysis of the above mentioned factors.

The compounds have antibacterial activity, in particular, against widely diffused gram positive bacteria and are particularly useful for topical treatment of wound infections and acne.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
Y	THE JOURNAL OF ANTIBIOTICS, vol. 37, no. 4, 1984, pages 309-317, Tokyo, JP; B. CAVALLERI et al.: "A-16686, a new antibiotic from Actinoplanes. 1. Fermentation, isolation and preliminary physico-chemical characteristics" * Pages 310,311,312,315,317 * ---	1-4	C 07 K 11/02 A 61 K 34/02
A	IDEM ---	5	
Y	THE JOURNAL OF ANTIBIOTICS, vol. 40, no. 1, 1987, pages 49-59, Tokyo, JP; B. CAVALLERI et al.: "Teicoplanin, antibiotics from Actinoplanes Teichomyceticus nov. sp. VIII. Opening of the polypeptide chain of teicoplanin aglycone under hydrolytic conditions" * Pages 49,51,57 * -----	5	
			TECHNICAL FIELDS SEARCHED (Int. Cl.4)
			C 07 K C 12 P

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	24-04-1990	PEETERS J.C.

CATEGORY OF CITED DOCUMENTS

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